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Molecular methods confirm the first report of the non-indigenous *Perna viridis* Linnaeus, 1758 (Mytilida, Mytilidae) in southern Brazil

Mayara Carneiro Beltrão[1], Natalia Joana Rebello da Cunha[2], Yan de Oliveira Laaf[3], Fernando Luiz Diehl[2], Tayse Demetro dos Santos[2]

1 *Community Ecology Lab, Polytechnic School, University of Vale do Itajaí, Itajaí, Brazil*
2 *Acquaplan Tecnologia e Consultoria Ambiental, Balneário Camboriú, Brazil*
3 *Polytechnic School, University of Vale do Itajaí, Itajaí, Brazil*

Corresponding author: *Mayara Carneiro Beltrão (mbeltrao@outlook.com)*

Abstract. The mussel *Perna viridis*, commonly known as Green Mussel, is native from the Indo-Pacific region and has been introduced in various countries around the globe. In Brazil, the species has already been recorded in Rio de Janeiro and Ceará states. With the aim of assessing the presence of mussels in the southern region of the country, 14 individuals were collected in the Paranaguá Estuarine Complex, Paraná. The mussels were found attached at a depth of 2 m on the artificial structure of Ponta do Poço Marina. The DNA was extracted using a commercial kit, the COI gene was amplified through PCR using the primers dgLCO-1490 and dgHCO-2198, sequenced by the Sanger method, and the species was identified through the BOLD Systems. The phylogenetic tree was built on the software MEGA 11 using 28 sequences from three *Perna* species. Therefore, the present study confirms the occurrence of *P. viridis* in Brazil through molecular identification for the first time and adds a second Brazilian state where it has been recorded. The Brazilian coastline provides optimal environmental conditions for the establishment and development of *P. viridis*.

Key words. DNA analysis, Green Mussel, invasive species, marine species, Paraná

Beltrão MC, da Cunha NJR, Laaf YO, Diehl FL, dos Santos TD (2024) Molecular methods confirm the first report of the non-indigenous *Perna viridis* Linnaeus, 1758 (Mytilida, Mytilidae) in southern Brazil. Check List 20 (4): 859–867. https://doi.org/10.15560/20.4.859

INTRODUCTION

Alien species are defined as those found outside their natural distribution area and that have the potential to spread in this new area, where they could not occur without anthropic introduction (Pyšek et al. 2020). When they harm the economy, environment, or human health, these species are classified as invasive (IUCN 2021). The mytilids are one of the main invasive alien groups recognized for causing several impacts. Their success stems from their capability in adapting to new environments, becoming well established and dominant in coastal areas (Micklem et al. 2016; Santos et al. 2023).

Once established, invasive mytilids demonstrate increased tolerance to environmental stress in comparison to taxonomically related native species (Lenz et al. 2011), consequently modifying ecosystem functioning and structure through competition, and altering food chains and nutrient cycling (Pyšek et al. 2020). These species also affect human well-being by harming water systems in industrial complexes and marine transport, as well as causing human health problems through food poisoning. This is because their filter-feeding behavior concentrates heavy metals and other contaminants (Freire and Marafon 2018; Siriwardena 2022; Turbelin et al. 2022).

In Brazil, the occurrence of nine invasive species of marine or estuarine bivalves has been documented, with seven recorded in the southern region of the country: the oysters *Isognomon bicolor* (C.B. Adams, 1845), *Saccostrea cucullata* (Born, 1778), *Crassostrea virginica* (Gmelin, 1791), and *Crassostrea gigas* (Thunberg, 1793) and the mussels *Mytilus galloprovincialis* Lamarck, 1819, *Leiosolenus aristatus* (Dillwyn, 1817), and *Perna perna* (Linnaeus, 1758) (Moura-Britto and Patrocínio 2005; Freire and Marafon 2018; Messano et al. 2019; Amaral et al. 2020; Belz et al. 2020; Teixeira and Creed 2020; Agudo-Padrón 2022; Stanski et al. 2022).

Among the species of invasive mussels in Brazil, *M. galloprovincialis* is an intertidal species, native to the Mediterranean and the Atlantic coasts of Europe and North Africa. This mollusk is widely distributed, occupying shores on every continent except Antarctica (McQuaid et al. 2015; Zardi et al. 2018). *Leiosolenus aristatus* is a small, endolithic and wood-boring species that often causes damage and deformities in other molluscs. This mussel is native to the Caribbean Sea and has been identified in the Atlantic, Mediterranean,

OPEN ACCESS

Academic editor: Igor Christo Miyahira
Received: 18 December 2023
Accepted: 26 June 2024
Published: 16 July 2024

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Caribbean, and Pacific regions (Simone and Golçalves 2006; Soriano and Salgado 2019). Brown mussel, *P. perna*, is widely distributed around the world, and along the Brazilian coast it is particularly abundant from Espírito Santo to Santa Catarina, and there has been extensive study of this species' origin in the country. According to Pierri et al. (2016), its presence in Brazilian territory predates colonization in 1500, suggesting that *P. perna* is a native species. However, as demonstrated by Silva et al. (2018), the occurrence of the species along the Brazilian coast is attributed to invasion during the slave trade between Africa and Brazil from the 16th to the 19th centuries. Despite its origin, the species is naturalized in South America, with multiple introductions occurring over the past 500 years (Oliveira et al. 2017).

Although the presence of *P. viridis* has not yet been confirmed by molecular methods in Brazil, Messano et al. (2019) reported the first occurrence in the Southwest Atlantic. Green mussels were found in May 2018 attached to experimental plates installed to test anti-fouling systems at Guanabara Bay, Rio de Janeiro, on the southeastern Brazilian coast. Recently Santos et al. (2023) reported *P. viridis* in lantern nets in the Marine Extractive Reserve at Arraial do Cabo (Rio de Janeiro). Santos et al. (2023) suggested that all these introductions on the Brazilian coast were accidental, probably via fouling or ballast water.

Perna viridis inhabits the mesolittoral and sublittoral zones of marine and estuarine ecosystems, where it is usually found forming dense colonies attached to a variety of structures or substrates via byssus threads (Rajagopal et al. 2006). Over the years, it has been reported in polluted and anthropogenic areas (Rajagopal et al. 1997; Micklem et al. 2016), but also in pristine and protected systems (Gracia and Rangel-Buitrago 2020; Santos et al. 2023).

The species is native to the Indo-Pacific region, extending from the Persian Gulf to the Philippines and in adjacent areas, such as China and Japan (Baker et al. 2007). This mussel species has been introduced to various regions around the globe, mainly through ship-hull fouling, and its occurrence has been documented in the Northwest and Northeast Pacific, Oceania, South Africa, the Gulf of Mexico and the Caribbean Sea, and the Northwest Atlantic (Santos et al. 2023). In the early 1990s, the species was recorded being introduced in Trinidad and Tobago (West Atlantic) and subsequently dispersed to Jamaica, Venezuela, Colombia, and the United States by the end of the decade (Baker et al. 2007). Where this species has been introduced, it can cause significant negative impacts, including competition for space leading to displacement of native species and alterations in community structure (Gracia et al. 2011; Baker et al., 2012). Additionally, there is a risk of transmitting diseases and parasites to local species, as well as harming other aquaculture species, such as crabs and mussels by obstructing traps and cultivation bags (Gracia et al. 2011).

The exceptionally invasive capacity of *P. viridis* is primarily attributed to their physiology, which is characterized by a high rate of dispersion, phenotypic plasticity, rapid growth and recruitment, the ability to detach and re-attach with byssus, and the formation of dense clusters that alter the native ecosystem (Gobin et al. 2013). In addition to these characteristics, one of the main reasons for the competitive superiority of *P. viridis* is its high resistance to environmental stress. Lenz et al. (2011) compared Brachidontes exustus (Linnaeus, 1758), a native, Caribbean species, with *P. viridis* and concluded that under stressful conditions the non-native *P. viridis* exhibits a wider tolerance range. *Perna viridis* tolerates a high level of particulate matter, a water temperature range of 15 °C to 32.5 °C, and it can even survive at a water temperature of 39 °C for about 200 minutes (Rajagopal et al. 2006).

Perna viridis is recognized in the literature as a green-shelled mussel, with the color being one of the taxonomic characteristics used for its identification, but this is not a reliable character. Micklem et al. (2016) concluded that specimens genetically identified as the variants of indigenous *P. perna* in South Africa displayed a wide range of shell colors, including blue-green. This finding indicates that the designations *P. viridis* as "Green Mussel" and *P. perna* as "Brown Mussel" are unsuitable for discerning these species. The only morphological differences found to separate the taxa in the study were the poorly developed mantle papillae and the wavy pallial line in *P. viridis*. Thus, the exclusive reliance on morphological characteristics can present challenges in identification. In certain cases, species are indistinguishable based on morphology alone, therefore, the use of DNA assumes significant relevance as a tool to confirm the species identification (Smith et al. 2022). In this context, we aim to confirm the presence of a *P. viridis* in southern Brazil using molecular methods, and compile information about this species' expansion in the South Atlantic Ocean.

METHODS

The subtropical Paranaguá Bay Estuarine Complex, located in the extreme east of Paraná state, Brazil, encompasses an area of 612 km^2, making it the largest estuarine system in the state (Lana et al. 2001). The region, bordered by mangroves, marshes, and extensive tidal flats, exhibits seasonal variation patterns of water circulation and stratification (Knoppers et al. 1987; Egres et al. 2012; Moreira-González et al. 2020). The estuary has an average depth of 5.4 m and water residence time of 3.49 days (Lana et al., 2001). The salinity varies from 14.6 to 30.0 and temperatures range between 16.9 °C and 29.3 °C (Lana et al. 2001). This estuary complex experiences significant freshwater input, especially during the summer rainy season (December to May) when freshwater discharge is five times higher than in the winter dry season (June to

November) (Mantovanelli et al. 2004). The region has considerable ecological and economic importance for Brazil (Bumbeer and Rocha 2016) and has numerous nautical facilities, including marinas and ports. Notably, the Port Complex of Paraná represents the largest grain port in Latin America and the second largest public port in the country (Menem et al. 2019; Portos do Paraná 2023).

To confirm the species identification, on 15 February 2023, 14 individuals possibly belonging to *P. viridis* were sampled at a depth of 2 m on the artificial structures of Ponta do Poço Marina Club (25°32′55″S, 048°23′19″W) (Figure 1). In the sampling area, some of the collected individuals formed agglomerates (clusters) composed exclusively of the target species of the study, while others were sparsely dispersed. Entire specimens, including shells and tissues, were carefully detached from the artificial substrata using a metal spatula, placed in plastic containers, and transported on ice to the laboratory, where they were stored in a freezer.

The extraction of genomic DNA from the muscle of only one mussel sample was carried out using the commercial DNeasy Blood & Tissue kit (Qiagen, Germany). Subsequently, the DNA was quantified via spectrophotometry on the NanoQuant Infinite M200 Pro equipment (Tecan, Switzerland). The DNA was then used as a template for PCR amplification of the gene encoding for cytochrome oxidase subunit I (COI). For this, the primer forward dgLCO-1490 (5′-TAAACTTCAGGGTGACCAAARAAYCA-3′) and reverse dgHCO-2198 (5′-GGTCAACAAATCATAAAGAYATYGG-3′) were used, according to Meyer (2003). The 25 µl PCR reaction consisted of: 1× SuperFi II Buffer (ThermoFisher Scientific), 0.2 mM dNTP, 0.5 mM of each primer, 1U Invitrogen Platinum SuperFi II DNA Polymerase (ThermoFisher Scientific), 50 mg genomic DNA, and the final volume was adjusted with ultrapure water. The PCR reaction was conducted on the thermocycler (Veriti, Applied Biosystems), with the program: 94 °C for 2 min, 35 cycles of 94 °C for 15 sec, 58 °C for 30 sec, and 72 °C for 30 sec, with a final extension of 72 °C for 2.5 min. To confirm the amplification, the PCR products were evaluated through agarose gel electrophoresis (1%) and compared with the molecular marker DNA (λ Hind III, Invitrogen). The fragments were sequenced by the GoGenetics company, in both directions using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA), with the mentioned primers and the product evaluated on the ABI 3500x Genetic Analyzer (Applied Biosystems, USA) as described by the manufacturer.

The sequencing results were processed using the bioinformatics package CLC Genomics Workbench v. 6.5.2 (QIAGEN, Denmark). Initially, the sequences obtained in both directions were subjected to a step of eliminating low-quality segments (<Q30) using the Trim tool. Then, the shared regions between the two sequences were recognized and overlapped to obtain a single continuous segment of the COI gene, using the DNA Assemble tool. Finally, the obtained segment was then analysed on the Barcode of Life Data Systems (BOLD Systems) v.4 (Ratnasingham and Hebert 2007) portal in order to carry out the first molecular identification step. The sequence was compared to those available in the Species Level Barcode Records database. In this database, every COI barcode record with a species-level identification and a minimum sequence length of 500 base pairs (bp) was included.

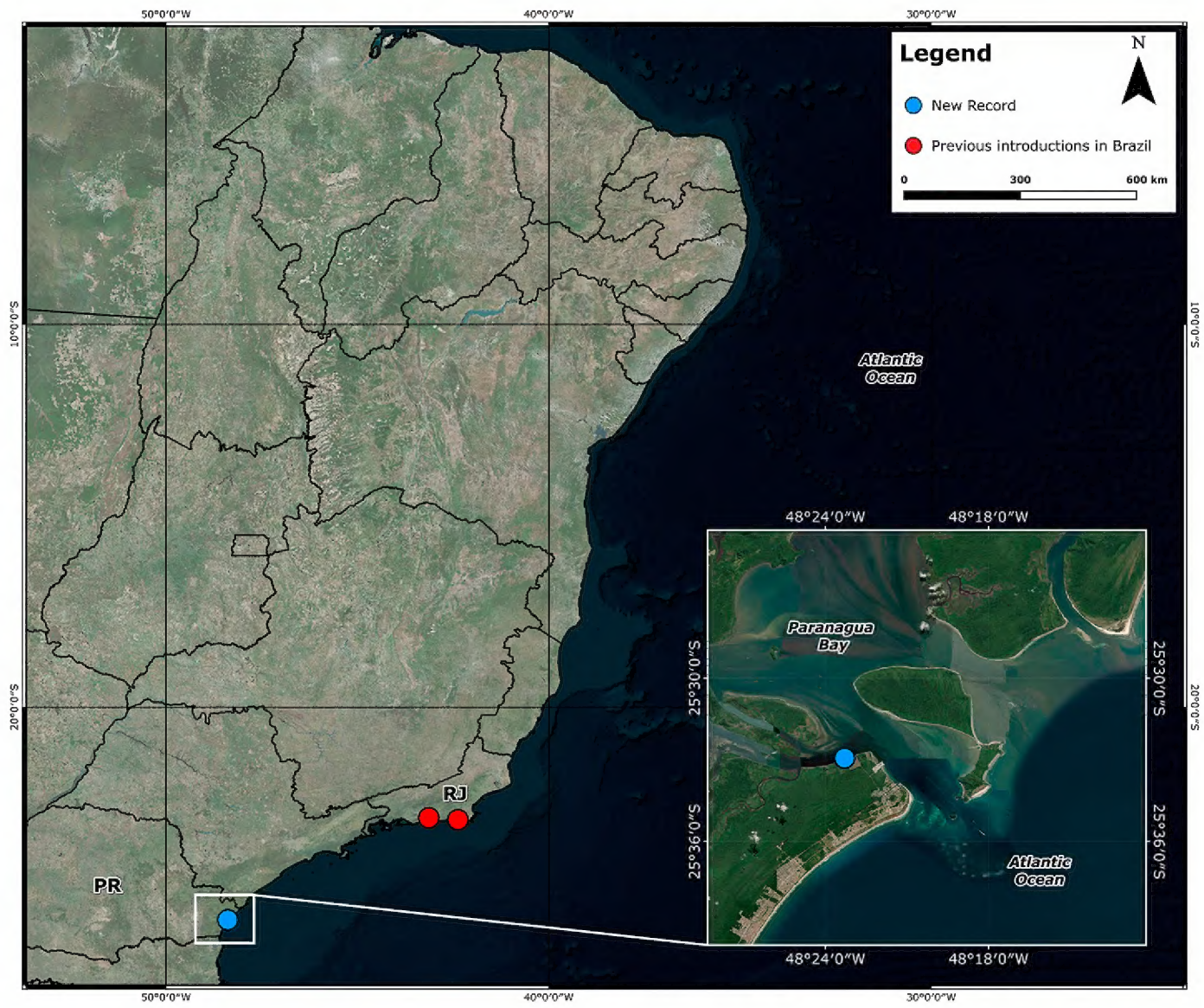

Figure 1. The range expansion of *Perna viridis* in Brazil. Red dots indicate areas where the mussel has already been recorded, and a blue dot marks the location of the new record in the Paranagua Bay Estuarine Complex, southern Brazil. RJ, Rio de Janeiro. PR, Parana.

The second molecular identification of the mussel was done through a comparative analysis of its COI sequence and that of other specimens within the same genus, including the same species. This was done by searching for published COI sequences in the NCBI's GenBank nucleotide database that also had the location in which they were sampled (Table 1). Then, sequences were aligned using the ClustalW algorithm available in MEGA11 software. The maximum-likelihood ultrametric phylogenetic tree was then built in the same software, with sequences from *P. perna*, *P. viridis*, and *P. canaliculus* (Gmelin, 1791), using the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985), and using *Aulacomya atra* (Molina, 1782) as an outgroup. The test was made with the bootstrap method, using 1000 replications.

RESULTS

***Perna viridis* (Linnaeus, 1758)**
Figures 2, 3

New records. BRAZIL – Paraná • Paranaguá Estuarine Complex, Ponta do Poço Marina Club; 25°32′55″S, 048°23′19″W; 2 m deep; 15.II.2023; E.I. Xavier leg.; artificial substrata; 14 spec.; metal spatula; Universidade do Vale do Itajaí (UNIVALI) collection (mol.PV1/PR).

Table 1. COI sequences of *Perna* species and *Aulacomya atra* used in the phylogenetic tree, organized by species name, collection locality, GenBank accession number, nucleotide length, and references.

Species	Locality	GenBank accession	Nucleotide length (bp)	Reference
P. viridis	Paraná, Brazil	PP702447.1	608	This study
	Kochin, India	JN179068.1	650	Gilg et al. 2013
	Kochin, India	JN179072.1	650	Gilg et al. 2013
	Hong Kong	GQ497818.1	650	Gilg et al. 2013
	Hong Kong	JN179047.1	650	Gilg et al. 2013
	Singapore	HQ197379.1	650	Gilg et al. 2013
	Singapore	JN179049.1	650	Gilg et al. 2013
	Chennai, India	DQ917612.1	617	Wood et al. 2007
	Southern India	DQ917586.1	617	Wood et al. 2007
	Philippines	DQ917599.1	617	Wood et al. 2007
	Thailand	DQ917590.1	617	Wood et al. 2007
	Vietnam	DQ917584.1	617	Wood et al. 2007
P. perna	Luanda, Angola	KC692001.1	614	Cunha et al. 2014
	Punta D'Ouro, Mozambique	KC692009.1	614	Cunha et al. 2014
	Swakopmund, Namibia	KC692005.1	614	Cunha et al. 2014
	Muscat, Oman	KC692013.1	614	Cunha et al. 2014
	Gans Bay, South Africa	KC691990.1	614	Cunha et al. 2014
	Bizerte, Tunisia	KC691986.1	614	Cunha et al. 2014
	Africa	DQ917618.1	617	Wood et al. 2007
	Venezuela	DQ917588.1	617	Wood et al. 2007
	Santa Catarina, Brazil	DQ917594.1	617	Wood et al. 2007
	São Paulo, Brazil	DQ917592.1	617	Wood et al. 2007
P. canaliculus	Houhora, New Zealand	DQ917607.1	617	Wood et al. 2007
	Castlepoint, New Zealand	DQ917613.1	617	Wood et al. 2007
	Gore Bay, New Zealand	DQ917608.1	617	Wood et al. 2007
	Fiordland, New Zealand	DQ917609.1	617	Wood et al. 2007
	Nelson, New Zealand	HG005373.1	706	Pochon et al. 2013
Aulacomya atra	Wellington, New Zealand	DQ917614.1	620	Wood et al. 2007

Figure 2. Left valve of *Perna viridis* specimen collected in Paranaguá Estuarine Complex, Paraná, Brazil, and identified by COI sequence.

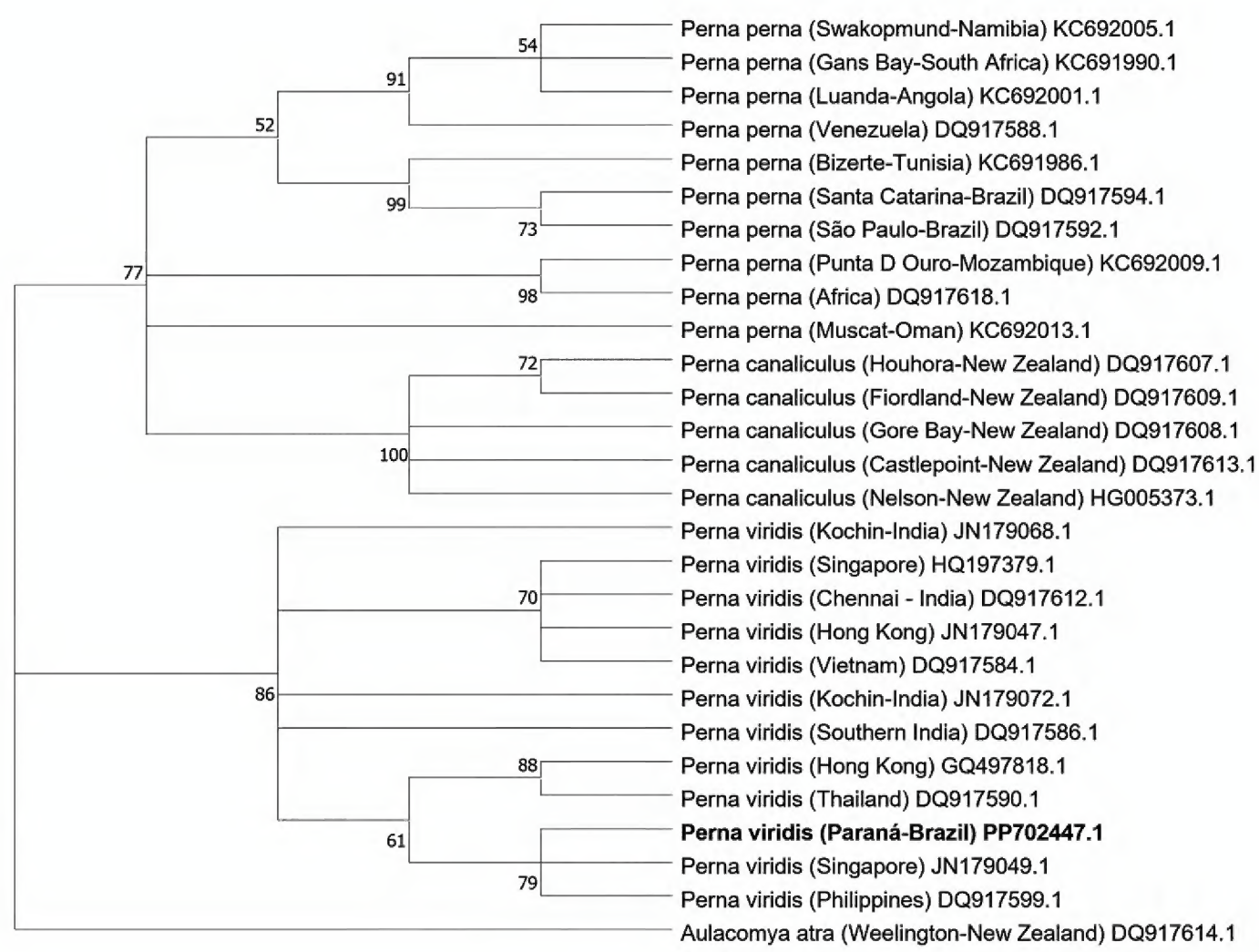

Figure 3. Maximum-likelihood phylogenetic tree (HKY+G+I) of various species within the genus Perna. Each node represents a different specimen's COI sequence, with its GenBank accession number and the locality from where it was sampled. Each branch has a label that represents its bootstrap percentage. Branches with <50% bootstrap value were collapsed. The sequence highlighted in bold was generated in this study.

Identification. Shell externally green or bluish-green; mantle papillae poorly developed; posterior pallial line wavy and S-shaped; beak downward-pointing.

Through the comparison of the mitochondrial gene COI from the mussel collected in Paranaguá Bay Estuarine Complex (*Perna viridis*, GenBank PP702447.1) with sequences from all three species within the *Perna* genus, obtained from NCBI's nucleotide bank, we confirm that our specimens correspond to the exotic mussel *Perna viridis* (Linnaeus, 1758) (Figures 2, 3).

DISCUSSION

Based on the amplified COI gene, we confirm here the occurrence in Brazil of the exotic *Perna viridis* through molecular identification. Our new record represents the third Brazilian state where this mussel species has been recorded and expands this species' range by approximately 600 km south of the first instance recorded five years ago. This species has already been identified in the southeastern Brazil (Messano et al. 2019; Santos et al. 2023) and now its occurrence has been documented in the southern region, indicating that the country's coast presents optimal environmental conditions for the establishment and growth of the mussel. According to Siddall (1980), *P. viridis* has the capacity to disperse beyond its native range through stepwise larval dispersal or island hopping. However, due to the absence of rafting skills, mytilids require vectors to move over long distances (Gracia and Rangel-Buitrago 2020). Due to these characteristics, ship ballast water and hull fouling have been identified as the main sources of the species' introduction (Bumbeer and Rocha 2016; Siriwardena 2022; Castro et al. 2023; Santos et al. 2023).

Port operations represent one of the most significant impacts on coastal environments worldwide, leading to alteration of aquatic ecosystems and the increasing presence of non-indigenous species (Madon et al. 2023). Regardless of the Paranaguá Estuarine Complex having a status of a preserved coastal environment (UNESCO 2014), the region is exposed to numerous anthropogenic impacts, including port activities that may facilitate the invasion of species. This trend was as noted in previous investigations in the area, which have indicated that the port acts as an entry point for non-indigenous species. Neves et al. (2007) identified in a marina near Paranaguá Port the presence of four introduced species and another 30 cryptogenic species. In Bumbeer and Rocha's (2016) study, which compared the Paranaguá Estuarine Complex and the adjacent open coast, the percentage of non-indigenous species was more pronounced in the estuary. Rocha and Kremer (2005) confirmed that the area is susceptible to non-indigenous species invasion.

Therefore, considering the potential for bioinvasion in the Paranaguá Estuarine Complex, primarily due to port activity, associated with the dispersal characteristics of *P. viridis* and the distances between records of the species' occurrence in Brazil, the most likely means of mussel introduction in the study area is accidental—via ballast water or ship hull fouling—rather than natural dispersal from the southeastern Brazil.

Despite extensive discussions surrounding invasive species, this issue is globally acknowledged as the second largest threat impacting biodiversity, and its negative consequences are generally irreversible (Loehle and Eschenbach 2012; Latini and Resende 2016; Stanski et al. 2022). Therefore, following Pyšek et al.'s (2020) proposal, we suggest that an integrated national invasive alien species surveillance program be implemented on the Brazilian coast and that monitoring along the Southwest Atlantic coast be continued to verify the range expansion of *P. viridis*. Future steps should include advancing the discussion about the recruitment and growth of *P. viridis* populations, as well as interactions and impacts on native species in places where their occurrence has been confirmed. This is important because the influence of alien species varies notably across species, regions, and ecosystems (Blackburn et al. 2014).

ACKNOWLEDGEMENTS

We thank Emerson Ivonésio Xavier for his invaluable assistance in the field.

ADDITIONAL INFORMATION

Conflict of interest

The authors declare that no competing interests exist.

Ethical statement

No ethical statement is reported.

Funding

This study was financially supported by Acquaplan Tecnologia e Consultoria Ambiental Group.

Author contributions

Conceptualization: MCB. Investigation: MCB, NJRC, YOL, TDS. Funding Acquisition: FLD. Project administration: MCB. Resources: FLD. Supervision: MCB. Validation: FLD. Writing – original draft: MCB, NJRC, YOL, TDS. Writing – review and editing: MCB, NJRC, YOL.

Author ORCID iDs

Mayara Caneiro Beltrão https://orcid.org/0000-0001-7634-7877
Natalia Joana Rebello da Cunha https://orcid.org/0000-0002-8741-7110
Yan de Oliveira Laaf https://orcid.org/0009-0003-5655-1223
Fernando Luiz Diehl https://orcid.org/0000-0002-6821-852X
Tayse Demetro dos Santos https://orcid.org/0009-0006-8575-7759

Data availability

All data that support the findings of this study are available in the main text.

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